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Cutinase Gene Disruption in *Fusarium solani* f sp *pisi* Decreases Its Virulence on Pea

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***Fusarium solani* f sp *pisi* (*Nectria haematococca*) isolate 77-2-3 with one cutinase gene produced 10 to 20% of the cutinase produced by isolate T-8 that has multiple cutinase genes, whereas cutinase gene-disrupted mutant 77-102 of isolate 77-2-3 did not produce cutinase. On the surface of pea stem segments, lesion formation was most frequent and most severe with T-8, less frequent and less severe with 77-2-3, and much less frequent and much milder with the gene-disrupted mutant. Microscopic examination of the lesions caused by the mutant strongly suggested that it penetrated the host mostly via the stomata. In seedling assays, 77-2-3 caused severe lesions on every seedling and stunted growth, whereas the mutant showed very mild lesions on one-third of the seedlings with no stunting. Thus, cutinase gene disruption resulted in a significant decrease in the pathogenicity of *F. s. pisi* on pea.**

INTRODUCTION

Many phytopathogenic fungi penetrate directly through the host cuticle to infect plants. Whether this penetration is assisted by enzymatic degradation of cutin, the structural polymer of the host cuticle, has been debated for the better part of a century (van den Ende and Linskens, 1974; Kolattukudy, 1985). Cutinase, the enzyme postulated to be involved, was first purified and characterized from *Fusarium solani* f sp *pisi* (*Nectria haematococca*) grown with cutin as the sole source of carbon (Purdy and Kolattukudy, 1975a, 1975b). That a cutinase, which immunologically cross-reacts with the enzyme produced under this saprophytic growth condition, is produced when *F. s. pisi* penetrates the host cuticle during infection was demonstrated using immunoelectron microscopy (Shaykh et al., 1977). Inhibition of cutinase using a variety of mechanism-based inhibitors as well as with specific antibodies has been found to prevent infection of pea stem with intact cuticle by *F. s. pisi*, but such agents show no effect on infection of the stem with breached cuticle (Maiti and Kolattukudy, 1979; Köller et al., 1982a, 1982b). In further support of the conclusion that assistance by cutinase is important to infection by highly virulent strains of *F. s. pisi*, a mutant of a highly virulent T-8 strain of *F. s. pisi* with reduced cutinase activity was found to have decreased virulence, and this virulence could be enhanced by the addition of cutinase to the inoculum (Dantzig et al., 1986). When the cutinase gene from *F. s. pisi* was transferred to *Mycosphaerella*, a papaya pathogen that infects only through wounds (Dickman et al., 1982), the transformants that inducibly expressed the *Fusarium* cutinase were found to be capable of infecting fruits with intact cuticle, and antibodies against the *Fusarium* cutinase prevented this infection (Dickman et al., 1989). Thus, many different lines of evidence show that the extracellular cutinase assists the highly virulent strains of *F. s. pisi* to infect their hosts.

Because the highly virulent strains of *F. s. pisi* have multiple cutinase genes (Kolattukudy et al., 1985), strain 77-2-3, with one cutinase gene, was used to generate cutinase gene–disrupted transformants (Stahl and Schäfer, 1992). It was reported that these transformants retained virulence, and therefore, it was concluded that cutinase did not play a significant role in pathogenesis. In this study, we describe results of a detailed analysis of virulence of the same cutinase gene–disrupted mutant on pea stem sections and pea seedlings and demonstrate that cutinase gene disruption significantly decreased the virulence.

RESULTS

Cutinase Inducibility in *F. s. pisi* Isolates T-8, 77-2-3, and the Cutinase Gene-Disrupted Mutant 77-102

Cutinase production induced by growth on cutin as the sole carbon source and by cutin hydrolysate in glucose-grown cultures of *F. s. pisi* isolates T-8, 77-2-3, and its cutinase gene–disrupted mutant 77-102 was tested. *p*-Nitrophenyl butyrate hydrolase activity was induced to very high levels in T-8, which is known to contain multiple cutinase genes as previously observed (Kolattukudy et al., 1985), whereas the wild-type 77-2-3, which probably contains only one cutinase gene (Stahl

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and Schäfer, 1992), produced much less hydrolase, as shown in Figure 1A; the gene-disrupted mutant produced hardly detectable levels of hydrolase activity. Induction by cutin hydrolysate in glucose-grown cultures gave similar results (data not shown). Immunoblot analysis of the culture fluids revealed only one band in both T-8 and 77-2-3 (Figure 1B). Even very long exposure of the blots did not reveal a band at the same size from the cultures of the gene-disrupted mutant.

The time course of production of immunologically detectable cutinase showed that the single gene–containing isolate produced very much less cutinase than that produced by T-8 (Figure 1C). ^{3}H-cutin hydrolase activity levels of the cultures reflected the *p*-nitrophenyl butyrate esterase activity and the immunologically detectable levels of cutinase (Figure 1C). The maximal level of *p*-nitrophenyl butyrate esterase activity, ^{3}H-cutin hydrolase activity, and immunologically detectable cutinase induced in the 77-2-3 cultures were < 10, 16, and 17%, respectively, of those produced by T-8. The culture fluid of the gene-disrupted mutant did not show measurable cutin-hydrolase activity.

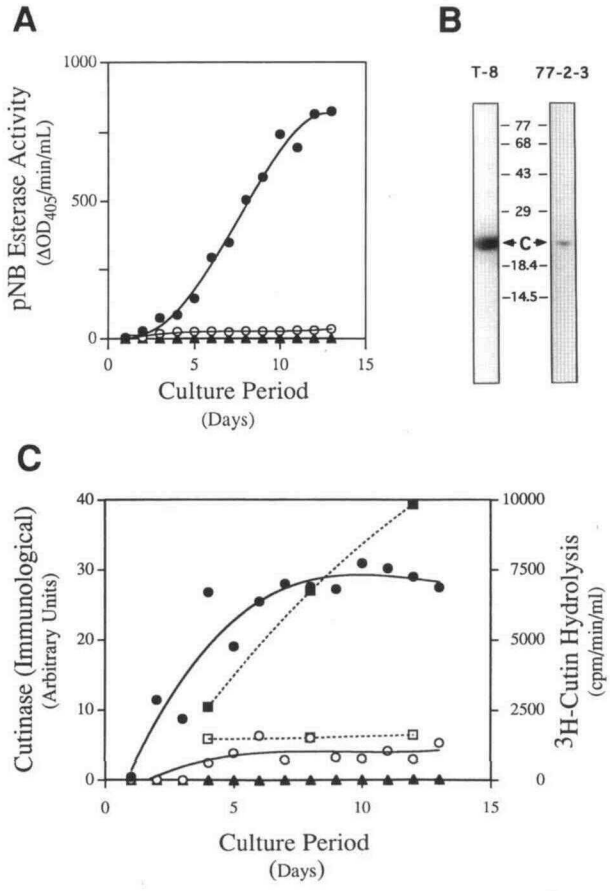

Figure 1. Cutinase Production by *F. s. pisi* Isolates and a Cutinase Gene–Disrupted Mutant.

(A) Induction of cutinase production in *F. s. pisi* isolates T-8 (●) and 77-2-3 (○) and cutinase gene–disrupted mutant 77-102 (▲) when grown on cutin, as measured by *p*-nitrophenyl butyrate (*p*NB) esterase activity. Although the difference between 77-2-3 and 77-102 is not clear as shown because of the low values, the actual OD_{405}/min/mL for the 13-day period are 34.9 and 1.2 for 77-2-3 and 77-102, respectively.

(B) Protein gel blot using antibody raised against *F. s. pisi* T-8 cutinase; the position of cutinase is indicated by C and arrows. The numbers in the middle show the positions of molecular mass markers in kilodaltons.

(C) Immunologically measurable cutinase production induced by cutin in isolates T-8 (●) and 77-2-3 (○) and mutant 77-102 (▲). ^{3}H-cutin hydrolase activities of T-8 (■) and 77-2-3 (□) are also shown. All procedures are as described in Methods.

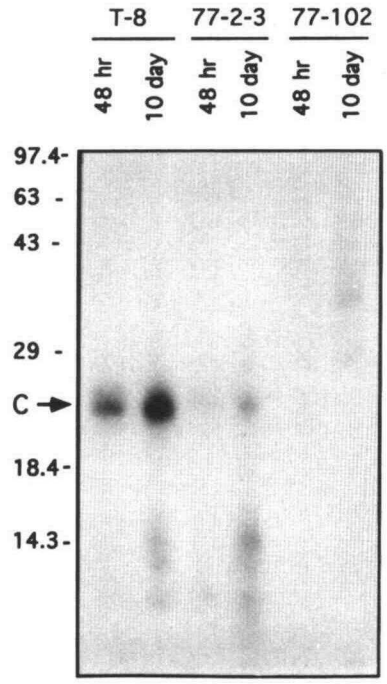

Figure 2. Labeling of Active Serines in Extracellular Proteins.

An autoradiogram of an SDS–polyacrylamide gel of ^{3}H-DipF–treated total extracellular protein from cutin-grown cultures of *F. s. pisi* isolates T-8 and 77-2-3 and cutinase gene–disrupted mutant 77-102 is shown. Spores (3.4×10^7) were used for inoculation of each culture; the period of culture growth is indicated at the top and molecular mass markers (Gibco-BRL) in kilodaltons are shown at left; the position of cutinase is indicated by C and the arrow. Because of the differences in total label incorporation, equal amounts of radioactivity (40K cpm) rather than equal amounts of protein were subjected to SDS-PAGE. All procedures are as described in Methods.

To test for the possible production of other active serine-containing cutinases by the *Fusarium* isolates or the gene-disrupted mutant, extracellular proteins were treated with ^{3}H-diisopropyl fluorophosphate (^{3}H-DipF), and the products were analyzed by SDS-PAGE and autoradiography, as shown in Figure 2. Only one cutinase could be found in the filtrate from the 48-hr culture of isolate T-8, and the same cutinase was found at much higher levels after 10 days. Some smaller molecular weight components, presumably representing proteolytic nicks, were also seen. Isolate 77-2-3 also showed the

same cutinase and lower molecular weight products. However, the amount of ^{3}H-DipF labeling of extracellular proteins showed that 77-2-3 produced only 5 to 11% of extracellular active serine enzymes produced by T-8. With the cutinase gene–disrupted mutant, ^{3}H-DipF–labeled cutinase could not be detected, although after 10 days small amounts of label were found in higher molecular weight components as previously found (Stahl and Schäfer, 1992). Because the amount of extracellular fluid from the mutant used for SDS-PAGE was 10 times that from the wild type, these higher molecular weight proteins are only minor components. In any case, the relative levels of cutinase revealed by ^{3}H-DipF labeling are in agreement with those shown by *p*-nitrophenyl butyrate esterase and ^{3}H-cutin hydrolase activities and immunological measurements. Thus, all of the results showed that the isolate 77-2-3 produced much lower levels of cutinase than the highly virulent T-8, and the cutinase gene–disrupted mutant had lost the ability to induce production of cutinase by cutin or cutin hydrolysate.

Cutinase Gene Disruption Limits Infection of Pea Stem Segments

Because the natural site of infection of *F. s. pisi* is just above the point of cotyledon attachment (Bywater, 1959; Kraft et al., 1981), only a single segment of etiolated pea stems from just above the cotyledon of each seedling was used for bioassays so that the fungus would be presented to the same region of the host in the bioassays as would occur in natural infections. In all cases, spore levels of 10^{3}, 10^{4}, 10^{5}, and 10^{6} were placed on the stem because the percentage of stem segments that showed lesions depended on the inoculum level; at a very high level (10^{6}) nearly all segments were infected, and differences in virulence tended to be obscured as previously noted (Köller et al., 1982c). The time course of lesion development revealed that most of the segments with 10^{6} spores began to develop visible lesions in 48 hr. The severity of lesions and the percentage of stem segments showing lesions increased more rapidly when higher levels of spores were introduced. In spite of variations in the frequency of infection, as shown in Table 1, daily observations with different levels of spores in four different experiments clearly showed differences in the degrees of virulence (Figure 3 and Table 1); T-8 was much more highly virulent than 77-2-3, and the cutinase gene–disrupted mutant consistently showed substantially less virulence than did the wild type.

Visual examination of lesion development by the three *F. s. pisi* cultures revealed that the gene-disrupted mutant resulted in much milder lesions that started as narrow brown lines within the area where the infection droplets were placed, whereas the wild type and T-8 resulted in more generalized lesions throughout the area covered by the infection droplets. To test whether the gene-disrupted mutant might have undergone coincident mutations that might have affected its virulence, we compared the virulence of the wild type and mutant on pea stems with mechanically breached cuticles. If such coincident mutations were responsible for the decreased virulence, the mutants should show the same decreased virulence

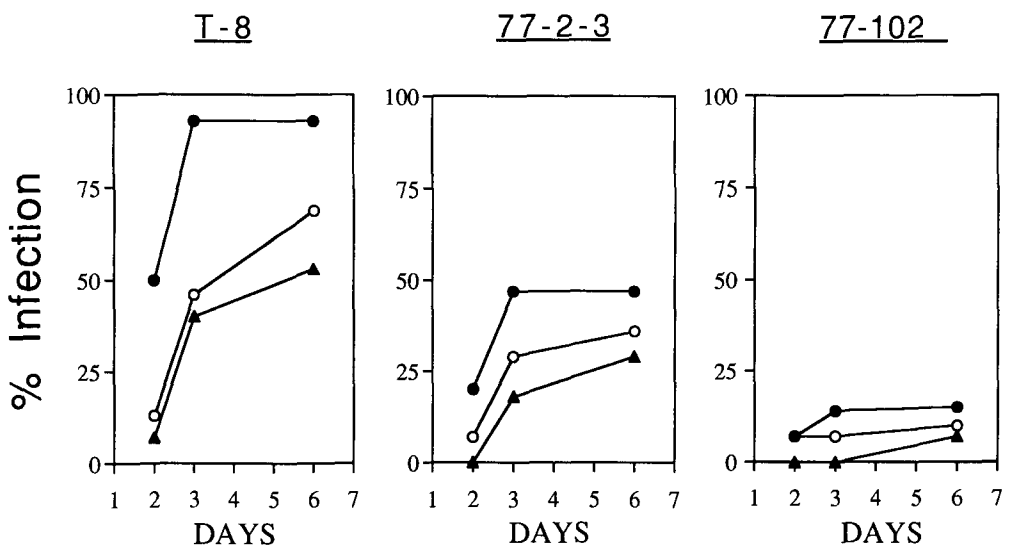

Figure 3. Time Course of Lesion Development in Pea Stem Bioassays.

Shown is the time course of lesion development when spore suspensions of *F. s. pisi* isolates T-8 and 77-2-3 and the cutinase gene–disrupted mutant were inoculated on pea stem segments for the periods indicated. Spores (10^{5} [●], 10^{4} [○], or 10^{3} [▲]) in 5-μL droplets were placed on each of 15 stems used for each data point.

Table 1. Frequency of Lesion Development on Pea Stem Sections by *F. s. pisi* Isolates T-8 and 77-2-3 and Cutinase Gene–Disrupted Mutant 77-102

Exp. No.	Days of Incubation	Spores/5-μL Droplet	% of Pea Stems Infected		
			T-8	77-2-3	77-102
1	3	10^5	60	36	20
		10^4	20	7	0
		10^3	0	0	0
	4	10^5	85	57	26
		10^4	47	14	13
		10^3	7	8	0
	5	10^5	100	67	30
		10^4	53	31	13
		10^3	21	9	7
2	6	10^5	72	31	18
		10^4	73	28	0
		10^3	67	0	3
3	3	10^5	69	15	7
		10^4	36	7	0
	4	10^5	100	46	7
		10^4	91	20	0

Pea stem infection assays were conducted as described in Methods.

level on stems with breached cuticles. However, on the host with mechanically breached cuticle the cutinase gene–disrupted mutant showed the same high degree of virulence as did the wild type, as shown in Table 2.

Cutinase Gene Disruption Limits Entry into Host to the Stomata

Microscopic examination of the lesion areas revealed that T-8 resulted in the most severe and generalized lesions with cell collapse, as shown in Figure 4A. Isolate 77-2-3 resulted in less severe but still generalized lesions with cell collapse (Figure 4B). However, the cutinase gene–disrupted mutant produced mild lesions localized in certain stoma-associated regions within the infection droplet area (Figure 4C). Figure 5A shows the host stem, where infection normally occurs, with an open stoma, and the lesion caused by the cutinase gene–disrupted mutant started at the stomata and was often confined to the stomatal region (Figure 5B). Staining of the lesion area showed penetration of the fungus at the stomata; brown lesions mostly at the stomata later spread as narrow brown lines from the stomata in a longitudinal manner (Figures 5C and 5D). This pattern was consistently observed in all of the experiments. Thus, the cutinase gene–disrupted mutant produced lesions in a smaller fraction of the stem segments, and the lesions were milder and localized at the stomatal regions.

Cutinase Gene Disruption Decreases Virulence on Pea Seedlings

We tested whether disruption of the cutinase gene also caused lowering of virulence on intact whole plants. We applied mutant and wild-type spores to vermiculite medium, and pea seeds were planted in the inoculated medium. The level of spores chosen was high enough to show a high degree of infection with the wild type. As the seedlings grew, the level of shoot growth was monitored. Generally, infection became visible by 8 to 12 days, as shown by the lower number of seedlings emerging from the medium and the lack of vigor in the growth of the shoots that emerged. Figure 6A shows representative seedlings grown in vermiculite inoculated with 77-2-3 and its gene-disrupted mutant 77-102 as well as uninoculated control plants. The overall growth of the latter two was indistinguishable, whereas infection was obvious with 77-2-3. Figure 6B shows all of the seedlings from a typical experiment. The seedling growth clearly demonstrated a significant reduction in virulence by cutinase gene disruption. Shoots of seedlings in the batch inoculated with the wild type were so badly infected that many of the seedlings hardly emerged from the medium, whereas such cases were extremely rare when inoculated with the gene-disrupted mutant (Figure 6B). The relative amounts of the biomass and especially the mass of the shoots also demonstrated differences in virulence, as shown in Table 3. For example, total weight of the biomass (shoot and root) of seedlings grown in the medium inoculated with the gene-disrupted mutant was 50% more than that of the seedlings inoculated with the wild type. The decrease in virulence was even more clearly seen in the weight of the shoots; the seedlings inoculated with the gene-disrupted mutant had 80% more mass than the shoots of the seedlings inoculated with the wild type.

Table 2. Frequency of Lesion Development on Intact and Mechanically Breached Pea Stem Sections by *F. s. pisi* Isolate 77-2-3 and Cutinase Gene–Disrupted Mutant 77-102

Days of Incubation	Spores/5-μL Droplet	% of Pea Stems Infected			
		77-2-3		77-102	
		Breached	Intact	Breached	Intact
3	10^5	93	19	100	0
	10^4	93	13	93	0
	10^3	93	0	76	0
4	10^5	93	20	100	0
	10^4	93	25	93	0
	10^3	93	0	94	0

Pea stem infection assays were conducted as described in Methods except that in the breached assay each stem was pricked one time with a sterile pin, and this breach was overlaid with 5 μL of spore suspension.

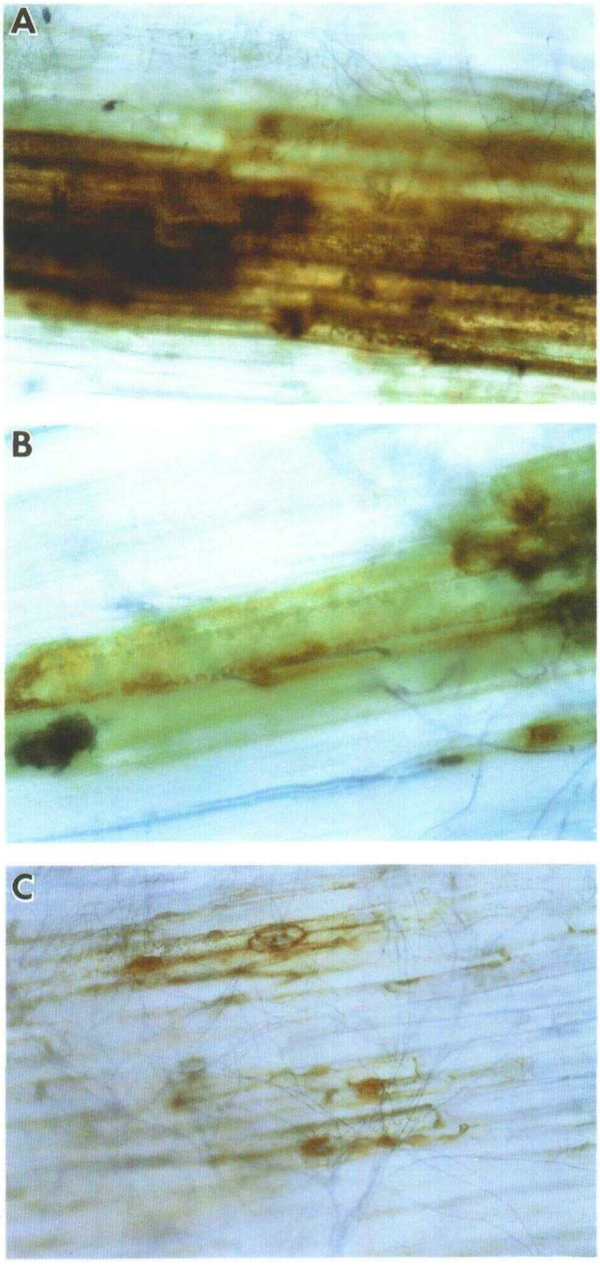

Figure 4. Microscopic Examination of Lesions on Pea Stems.

Photomicrographs of inoculated pea stem sections are shown.
(A) Section inoculated with *F. s. pisi* isolate T-8.
(B) Section inoculated with *F. s. pisi* isolate 77-2-3.
(C) Section inoculated with the cutinase gene–disrupted mutant 77-102.
Each stem received 10^6 spores in 5 μL of H_2O, and stems were microscopically examined after 6 days of incubation at 24°C. After examining many stem segments from four different experiments, we selected the representative segments that are shown. Procedures for the stem assay and microscopy are as described in Methods.

Lesion formation at the very base of the stem was found in most of the seedlings planted in the medium inoculated with spores of wild-type 77-2-3, whereas only less than one-third of the seedlings in the medium inoculated with the gene-disrupted mutant showed any lesions, as shown in Table 4. Even these limited numbers of lesions were invariably much smaller and milder. The stems from all of the seedlings from one experiment are shown in Figure 6C. Clearly, the severity of the lesions was dramatically less with the gene-disrupted mutant.

To ensure the identity of the inoculated cultures and to make sure that the fungi present in the medium at the end of the experiment truly represented the inoculated isolates, fungal cultures were obtained from the medium at the end of the experiment. Gel blots of the DNA isolated from these cultures and DNA from the original inoculum cultures were hybridized with cutinase cDNA. The wild-type 77-2-3 showed a single major hybridizing band with EcoRI, HindIII, PstI, and SstI, and the location of the band was shifted as expected as a result of the gene disruption (data not shown). Figure 7 shows the results obtained with KpnI, which demonstrates gene disruption as has been previously reported (Stahl and Schäfer, 1992) and shows that the isolates used for inoculation were the same as those present at the end of the experiment. As seen previously (Kolattukudy et al., 1985), isolate T-8 showed multiple cutinase bands, including a band corresponding to that found in the wild-type isolate 77-2-3 (Figure 7).

DISCUSSION

F. s. pisi infection is sometimes called root rot but as pointed out by others (Kraft et al., 1981), infection is more correctly called foot rot to indicate that the actual infection starts at the base of the stem at the cotyledonary attachment area. As a result, the first visible symptom appears as black flecks on the epidermis, and cytological studies indicate that the fungus appears in the epicotyl and hypocotyl before reaching the taproot (Bywater, 1959); the taproot is most probably not the primary site of entry of the pathogen. The results presented in this article clearly agree with such conclusions. Thus, the fungus in the soil penetrates the base of the stem that is covered by the cuticle. Chemical depolymerization and analysis of the products by combined gas liquid chromatography and mass spectrometry demonstrated that the cutin of the base of the stem is composed of 10,16-dihydroxypalmitic acid and 16-hydroxypalmitic acid (Maiti and Kolattukudy, 1979). Microscopic examination of the bases of the stems described in this paper clearly showed the presence of stomata in the region attacked by this pathogen. Thus, fungi should be able to penetrate either through the stomata or directly through the cuticle.

Obviously, the ability to penetrate through the cuticle that covers most of the surface would aid in the infection process. Therefore, *F. s. pisi* isolates capable of readily penetrating

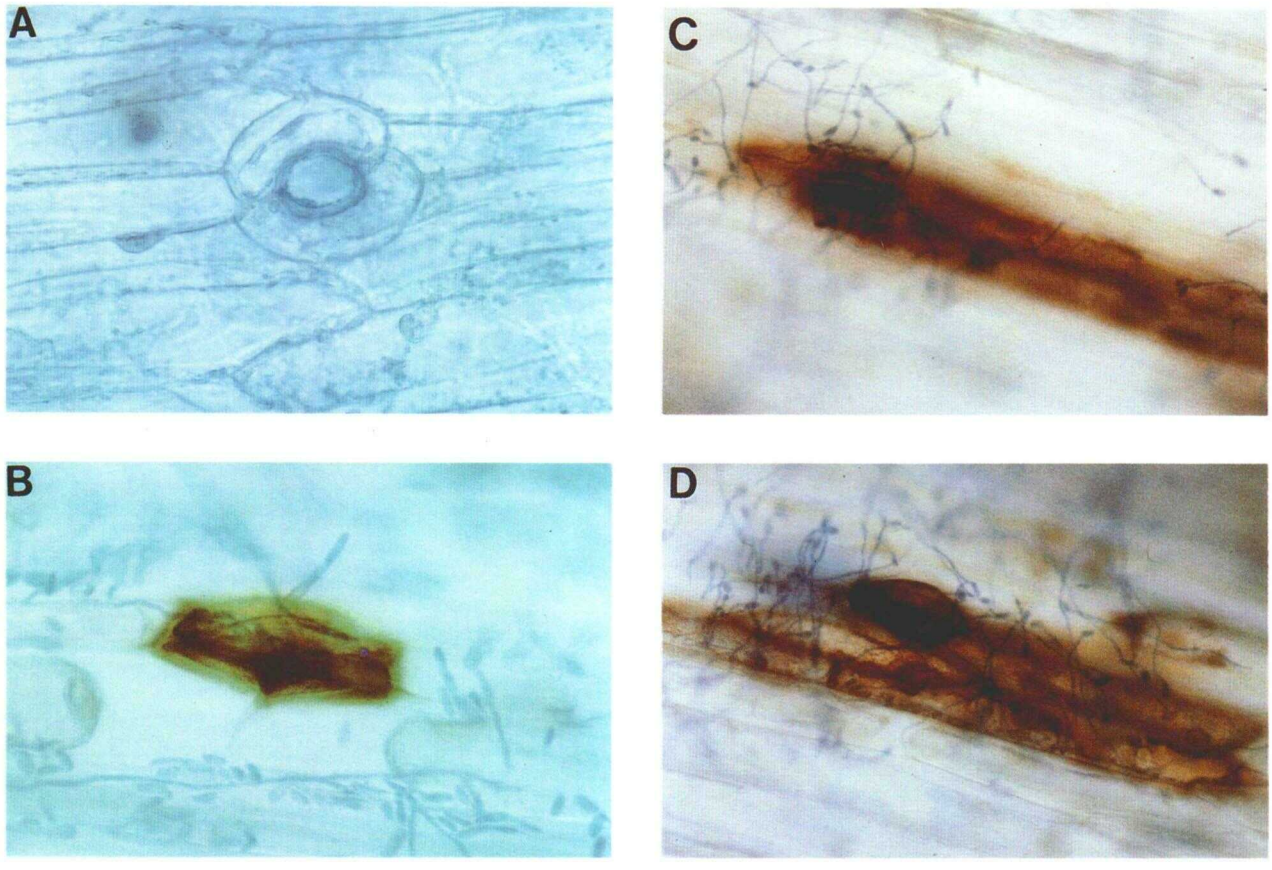

Figure 5. Microscopic Examination of Intact Pea Stems and Lesions Caused by Mutant 77-102.

(A) A stoma typical of those found in the pea stem 2 to 2.5 cm above the seed attachment in pea seedlings.
(B) to **(D)** Representative examples of progressive stages of infection of the pea stem by mutant 77-102.

through the cuticle would be expected to have higher virulence when compared to those that can enter the host mostly through the stomata. Thus, we have found that isolates capable of producing high levels of cutinase showed high virulence on tissue with intact cuticle (Köller et al., 1982c; Podila et al., 1989). A comparison of virulence of many *F. s. pisi* isolates (Miao et al., 1991) with their ability to produce cutinase upon growth on cutin as the sole carbon source (Kolattukudy et al., 1985) supports this conclusion, except for a few isolates that have high virulence on pea with breached cuticles but low virulence on intact hosts. Obviously, the ability to penetrate through the cuticle would not in itself make a virulent pathogen without the other capabilities needed for infection. However, an isolate with all such capabilities but incapable of penetrating through the cuticle (such as isolate T-30) would be less virulent on intact stems than isolates, such as T-8, that can penetrate through the cuticle; both could be equally virulent on stems with breached cuticles. In fact, isolate T-30 was found not to be virulent on intact stems but showed as high a virulence as T-8 on stems with pinpricks to breach the cuticle wall barrier (Köller et al., 1982c).

Isolates with a high degree of virulence on intact tissue produce high cutinase levels and have multiple cutinase genes (Kolattukudy et al., 1985); therefore, simple gene disruption cannot be done on such high-virulence isolates, although the role of cutinase would be expected to be most significant in the infection by such isolates. As the results presented in this article demonstrate, isolate 77-2-3, which probably contains one copy of the cutinase gene, produced much less cutinase and was much less virulent in pea stem bioassays than isolate T-8, which has multiple cutinase genes and produced very high levels of cutinase. Both the frequency of lesion formation and the severity of lesions support this conclusion.

Even with the low virulence of 77-2-3, the cutinase gene–disrupted mutant revealed much less virulence, as shown by the lower frequency of lesion formation on a pea stem with an intact cuticle. This decreased virulence was clearly demonstrated by the assays used in this study; these assays involved different

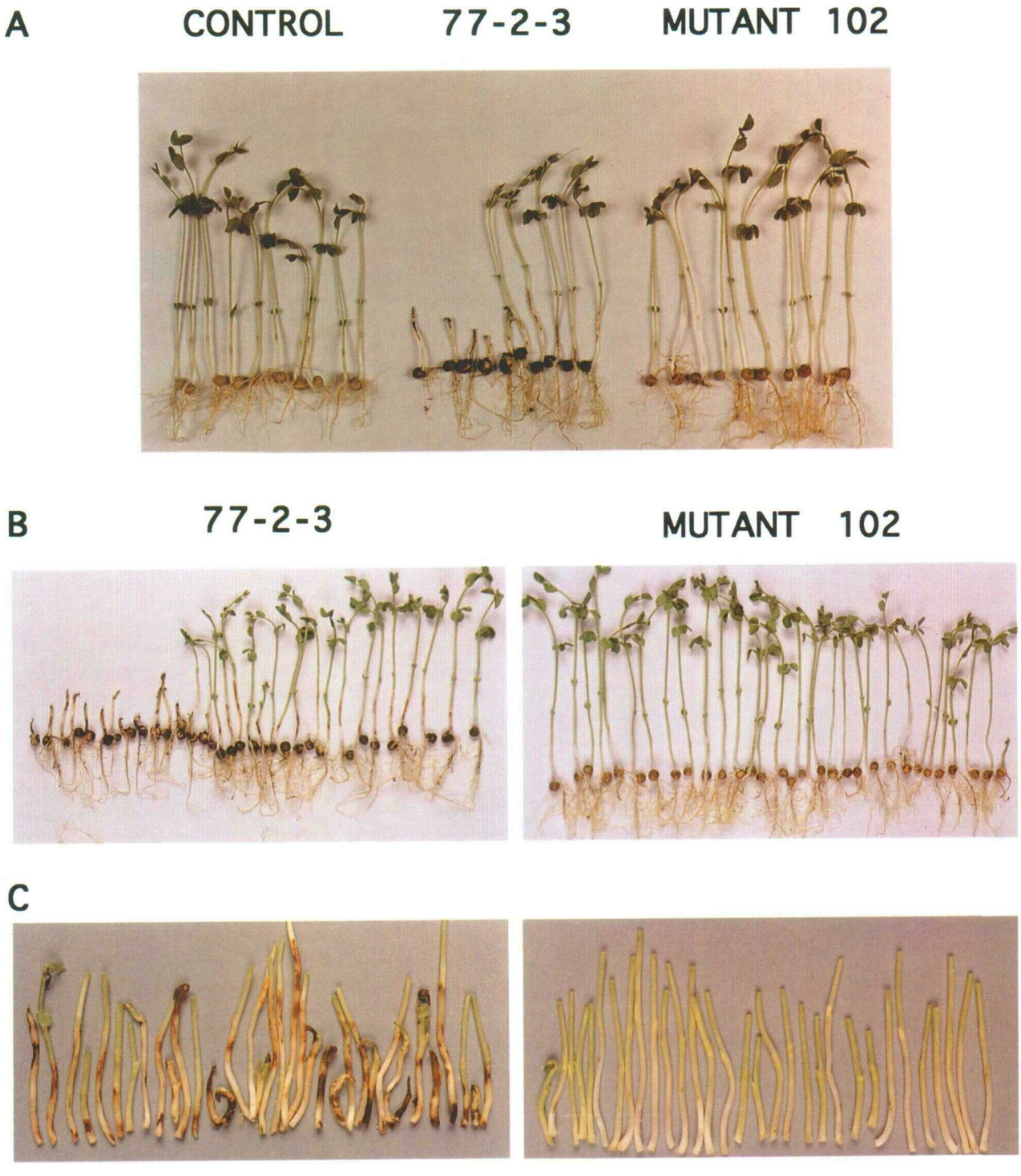

Figure 6. Infection of Pea Seedlings by *F. s. pisi* 77-2-3 and Cutinase Gene–Disrupted Mutant 77-102.

(A) Representative intact seedlings of an uninoculated control and seedlings inoculated with 77-2-3 or its gene-disrupted mutant 77-102.
(B) All seedlings from one experiment in which seedlings were grown on vermiculite inoculated with 77-2-3 or its mutant 77-102.
(C) The stems of seedlings inoculated with 77-2-3 (left) or its mutant 77-102 (right). Stems inoculated with 77-2-3 show severe lesions, and those inoculated with mutant 77-102 exhibit a limited number of lesions.

Table 3. Biomass of Pea Seedlings Grown on Vermiculite Inoculated with Spores of *F. s. pisi* Isolate 77-2-3 or Cutinase Gene–Disrupted Mutant 77-102

Exp. No.	Plant Organ	Biomass (g/plant) 77-2-3	Mutant 77-102
1	Shoot	0.25	0.45
2	Shoot and root (without cotyledon)	0.56	0.84

Experiment 1 included 36 plants infected with 77-2-3 and 31 plants infected with 77-102 that were all harvested 10 days after planting in inoculated vermiculite. Experiment 2 included 30 plants infected with 77-2-3 and 29 plants infected with 77-102 that were all harvested 11 days after planting.

spore levels and observations on the progress of lesion development. As shown by the results presented here, such assays can detect differences in virulence. In a previous evaluation of virulence of this mutant, multiple spore levels were not tested and microscopic examination of the progression of lesions was not reported (Stahl and Schäfer, 1992). There was no evidence presented to indicate that the assay conditions and methods used by these investigators to test for virulence would have detected decreased virulence. Experimental variations in virulence would make it necessary to test multiple levels of spores to assess virulence.

Our microscopic examination of the lesions generated suggested that isolate 77-2-3 penetrated not only through stomata but also through the cuticle, thus revealing lesions with cell collapse in the base area of many of the infection droplets; however, this isolate showed much less virulence than T-8. On the other hand, even the low number of mild lesions caused by the cutinase gene–disrupted mutant were limited lesions that showed close association with stomata. The results clearly showed a lowering of virulence that was expected as a result of the limitation of penetration only through the stomata. Stomatal penetration of *F. s. pisi* had been previously observed (Bywater, 1959). It might be possible to overwhelm the host tissue with very high levels of inoculum; this might result in the entry of an adequate number of germinating spores through the stomata and lead to more frequent lesion formation. In such cases, if the progression of lesion formation is not carefully examined and only depends on an end-point examination, the lesions would have progressed to the point that the differences in virulence would be obscured. In fact, our experience with 10^6 and higher spore levels showed that virtually all of the stems are quickly infected and that the differences in virulence are often less evident. That the cutinase gene–disrupted mutant did not contain other coincident mutations that affected virulence was shown by the observation that the mutant showed the same high virulence as the wild type on pea stems with mechanically breached cuticles; the wild type had been shown to be highly virulent on hosts with mechanically breached cuticles (Miao et al., 1991). Thus, the results presented here clearly show that cuticular penetration was essentially abolished by cutinase gene disruption, leading to drastically decreased virulence on intact host tissue.

The virulence assessment on intact seedlings presented in this article also revealed a clear decrease in virulence by disruption of the cutinase gene. The frequency of infection was less than one-third of that observed with the wild type, and even in these small numbers of lesions, the severity was drastically less in the case of the gene-disrupted mutant. The location and nature of the lesions observed were very typical of what has been described previously (Kraft et al., 1981). As is typical for the *F. s. pisi* infection (Kraft et al., 1981), growth was significantly decreased when seedlings were inoculated with the wild-type *Fusarium* isolate 77-2-3, but inoculation with the identical number of spores of the cutinase gene–disrupted mutant of equal viability did not detectably affect seedling growth. Examination of the seedlings showed that only a small fraction of the seedlings inoculated with the mutant had any lesions, and even these lesions were very mild and limited to small regions and thereby not inhibiting seedling growth. Shoot growth is usually most dramatically affected by *Fusarium* infection (Kraft et al., 1981), and in our study, the loss of virulence caused by cutinase gene disruption was clearly seen by the shoot mass of the seedlings. That the observed infections were, in fact, caused by the fungal isolates used for inoculation was demonstrated by gel blot analysis of DNA isolated from fungi cultured from the medium at the end of the experiment. Furthermore, serial dilution assays confirmed that the viability of the spores of the isolates used was identical. Thus, there is no alternative but to conclude that cutinase gene disruption drastically decreased virulence of *F. s. pisi* isolate 77-2-3.

The clear decrease in virulence caused by cutinase gene disruption observed here occurred even though the *Fusarium* isolate, 77-2-3, that was used in this study has only one cutinase gene and produces much less cutinase than some of the other more highly virulent isolates, such as T-8, that have multiple cutinase genes and show higher virulence on intact host tissue.

Table 4. Lesion Formation on Pea Seedlings Grown in Vermiculite Inoculated with Spores of *F. s. pisi* 77-2-3 and Cutinase Gene–Disrupted Mutant 77-102

Exp. No.	% of Plants Showing Lesions 77-2-3	Mutant 77-102
1	97	29
2	83	27
3	100	29

Plants were grown as described in Methods. For experiment 1, seedlings were grown in Magenta boxes at 26°C. For experiments 2 and 3, seedlings were grown in Phytacon containers at 22°C during the night and at 25°C during the day.

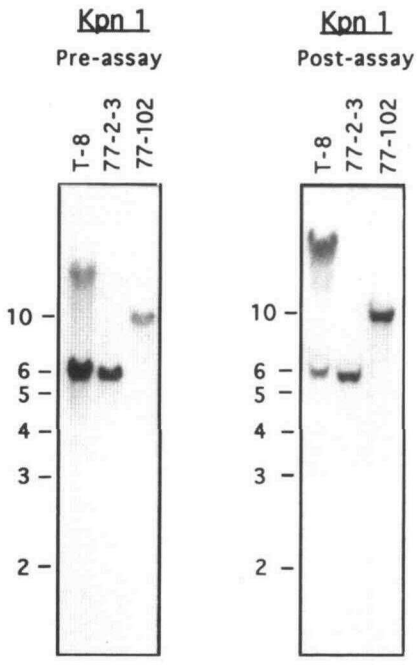

Figure 7. Genomic DNA Gel Blots for the Cutinase Gene.

DNA gel blots of KpnI-digested genomic DNA from *F. s. pisi* isolates T-8 and 77-2-3 and cutinase gene–disrupted mutant 77-102. Preassay refers to DNA from cultures used for inoculation, and postassay refers to fungal cultures from the vermiculite at the end of the experiment. T-8 is shown with the postassay for comparison. Molecular length markers (Gibco-BRL) in kilobases are shown at left. All procedures are as described in Methods.

With such highly virulent isolates, cutinase would be expected to play an even more important role in pathogenesis. Introduction of the cutinase gene from the highly virulent T-8 to isolate T-30 (which has a single gene, produces a very low level of cutinase, and has low virulence) enhanced the virulence of T-30; the transformants of T-30, which had cutinase levels approaching that of T-8, also showed highly enhanced virulence levels approaching that of T-8 (Kolattukudy et al., 1989). This enhancement of cutinase production leading to higher virulence and our results that show decreased virulence by cutinase gene disruption strongly support the conclusion that cutinase plays a significant role in the infection of peas by *F. s. pisi*. In another gene disruption study that questioned the role of cutinase in fungal penetration, the cutinase gene–disrupted mutant of *Magnaporthe grisea* used for testing virulence retained much of the cutin hydrolase activity of the wild type (Sweigard et al., 1992); therefore, no valid conclusion could be drawn about the role of cutinase in pathogenesis from this study.

A combination of mechanical force and assistance from cutinase probably allows penetration of most fungi that enter the host through the cuticle. The relative role of cutinase in this process probably varies a great deal. In cases where the cuticle is very thin and the pathogen forms highly melanized appressoria that have the capability to apply significant mechanical force, assistance by cutinase could be minor. However, in many cases, cutinase plays such a significant role that infection can be prevented by inhibition of cutinase; this has been demonstrated in several cases (Kolattukudy et al., 1994) and includes protection of gerbera flowers from *Botrytis cinerea* infection by monoclonal antibodies raised against the *Botrytis* cutinase (Cavete, 1992). However, under field conditions fungal infection may involve penetration directly through the cuticle, stomata, and wounds. Even in such cases, it might be possible to control infection by the most virulent strains using antipenetrants that would inhibit cutinase.

METHODS

Fungal Strains

Fusarium solani f sp *pisi* isolates T-8 and 77-2-3 were obtained from H. Van Etten, University of Arizona, Tucson. The cutinase gene–disrupted mutant of 77-2-3, designated 77-102, was obtained from W. Schäfer, Institut für Genbiologische Forschung, Berlin. These isolates were maintained on potato dextrose agar plates containing 0.5% (w/v) ground pea stem (PPDA plates) (Crawford and Kolattukudy, 1987) at 24°C.

Cutinase Induction

To test for induction of cutinase in the three *F. s. pisi* isolates, spores of each were harvested in H_2O from two 6-day-old fungal plate cultures and used to inoculate 100 mL of mineral medium (Hankin and Kolattukudy, 1968) containing 1.5% glucose. After cultures were grown for 48 hr at 24°C with shaking at 200 rpm, 4 mL of the resulting fungal suspension was used to inoculate each of eight Roux bottles (VWR Scientific, Chicago, IL) with 100 mL of minimal medium containing 0.5 g of apple cutin powder (Walton and Kolattukudy, 1972). To test for cutinase production, at 24-hr intervals 1 mL of extracellular medium was removed from each bottle; the eight samples from each set were pooled and assayed spectrophotometrically for esterase activity using *p*-nitrophenyl butyrate (Sigma) as the substrate (Purdy and Kolattukudy, 1973).

^{3}H-Cutin Hydrolysis by Extracellular Fluid

Appropriate volumes of extracellular fluid were incubated with 4 mg ^{3}H-cutin, prepared as previously described (Purdy and Kolattukudy, 1975a), in a total volume of 1 mL of 50 mM glycine-NaOH buffer, pH 10.0, for 10 min at 30°C in an orbital shaking water bath at 80 rpm. At the end of the incubation, reaction mixtures were quickly filtered through tightly packed glass wool, the filtrate was centrifuged 2 min in a microcentrifuge (Eppendorf, model 5415; VWR Scientific) to pellet any remaining fine particles of ^{3}H-cutin, and 0.4 mL of the supernatant was assayed for ^{3}H in Scintiverse II-BD (Fisher, Cincinnati, OH) using a liquid scintillation counter (model no. LS3801; Beckman Scientific, Fullerton, CA) at 60% counting efficiency.

Protein Gel Blot

A 4-mL aliquot of each extracellular fluid sample was dialyzed against H_2O and lyophilized; the residue was redissolved in 0.5 mL of H_2O. Aliquots (25 μL) were subjected to SDS-PAGE (Laemmli, 1970) with a 12.5% resolving gel, and the separated proteins were transblotted onto a Nytran Plus membrane (Schleicher & Schuell), blocked with 5% nonfat dry milk in 50 mM Tris-HCl, pH 7.2, with 200 mM NaCl and probed with rabbit antibody raised against cutinase from isolate T-8 (Soliday and Kolattukudy, 1976) and ^{125}I-protein A (10 μCi/μg; Du Pont–New England Nuclear). The iodine-125 in the immunoblot was quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

^{3}H-Diisopropylfluorophosphate Labeling of Extracellular Proteins

Roux bottles containing mineral medium with cutin were inoculated with 3.4 × 10^7 spores of each isolate. Extracellular fluid, which was collected by filtration of the culture through two layers of Whatman No. 1 filter paper after 48 hr and 10 days of growth, was concentrated to 50 mL by ultrafiltration using an Amicon PM-10 membrane (Amicon, Danvers, MA), dialyzed against water overnight at 4°C, lyophilized to dryness, and redissolved in 1 mL of H_2O. Aliquots were treated in 0.25 mL of 50 mM sodium phosphate buffer, pH 8.0, with ^{3}H-diisopropylfluorophosphate (^{3}H-DipF) (25 μCi) (4 Ci/mmol, Du Pont–New England Nuclear). After a 2-hr incubation at 24°C, the proteins were precipitated with 7.5% trichloroacetic acid and redissolved by boiling in Laemmli loading buffer (Laemmli, 1970). Aliquots (40,000 cpm) of each sample were electrophoresed as described above. The gel was treated with the autoradiography enhancer Fluoro-Hance (Research Products International, Mount Prospect, IL) according to the manufacturer's instructions, dried, and exposed to x-ray film.

Pea Stem Bioassay

Pea seeds (variety Extra Early Alaska; Livingston Seed Co., Columbus, OH) were surface sterilized with a 5% solution of Clorox (The Chlorox Company, Oakland, CA) for 1 to 5 min and washed for 2 hr under running H_2O. After soaking overnight in H_2O, peas were planted in a shallow layer of sterile vermiculite and grown for 5 to 7 days at 25°C in the dark. From each seedling, a 2- to 2.5-cm segment of the stem immediately above the cotyledon was excised for bioassays; all manipulations were done under sterile conditions. Spores from each isolate were harvested in H_2O from 5- to 10-day-old cultures grown on PPDA plates, filtered through sterile Miracloth (CalBiochem, San Diego, CA), washed twice with water by resuspension and centrifugation, and quantitated with a hemocytometer. The volume was adjusted to give 10^6, 10^5, 10^4, or 10^3 spores per 5 μL. Only microconidia were used for bioassays. Serial dilutions of the spore suspensions were plated on PPDA plates to determine relative viability.

For each sample, 15 stem sections were placed on H_2O-saturated filter paper in a sterile Petri dish, and 5 μL of the spore suspension was placed on the center of each section and incubated in a water-saturated container in the dark at 24 to 26°C. The moisture level of the assay chamber and Petri dishes was kept high by adding H_2O as necessary; spore droplets were not allowed to dry. Lesion development was monitored daily. Because of the variation in the frequency of lesion formation observed in four different experiments, the values are not averaged. Instead, the time course of lesion formation is shown in Figure 3, and frequency of lesion formation from three additional experiments is shown in Table 1; the frequency of lesion formation is expressed as the percentage of the total number of stem sections that showed lesions.

To microscopically examine the lesions, a thin layer of the stem with the infection area was excised and placed on a microscope slide under a coverslip. The stem section was stained with 50 μL of lactophenol-cotton blue for 5 min. After removal of excess dye, the stems were observed at 200× magnification with an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY); representative views were photographed using Ektachrome film (160T; Kodak).

Whole Plant Bioassay

Sterile vermiculite in either Magenta boxes (6 × 6 cm, 6-cm depth; 216-cm^3 volume; Sigma) or Phytacon containers (10-cm diameter, 13- to 14-cm depth; 850-cm^3 volume; Sigma) was thoroughly and evenly wetted with an appropriate volume of spore suspension, prepared as described above, and adjusted to 2 × 10^5 spores per mL; for Magenta boxes, 100 mL of spore suspension was needed, and for Phytacon containers, 400 mL was required. Surface-sterilized pea seeds (30 per container) were planted at ~3 cm deep into the vermiculite and incubated with a 15-hr day at various temperature regimes (24°C constant or 25°C day, 22°C night) for 10 to 12 days. Plants were watered periodically by the application of a misting spray. The seedlings were examined for lesions. Biomass of the seedlings and separate mass of the shoots and roots were determined. At the end of the experiment, the fungus was cultured on PPDA plates from the vermiculite and subsequently grown on glucose for DNA isolation.

Genomic DNA Gel Blots

Cultures of T-8, 77-2-3, and gene-disrupted mutant 77-102 used for inoculation of pea stems and fungal cultures obtained from vermiculite after seedling growth were grown for 48 hr on glucose as described above. Genomic DNA was prepared from the mycelia harvested by filtration as previously described (Kämper et al., 1994). Single-enzyme restriction digests of 10 μg of each DNA sample were electrophoresed on an 0.8% agarose gel and transferred by vacuum blotting to a Nytran Plus membrane. The blots were hybridized with ^{32}P-random primer–labeled *F. s. pisi* isolate T-8 cutinase cDNA (950 bp) (Soliday et al., 1984) and washed with 2 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4; Sambrook et al., 1989) containing 0.2% SDS at 37°C.

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REFERENCES

Bywater, J. (1959). Infection of peas by *Fusarium solani* var. Martii forma 2 and the spread of the pathogen. Trans. Brit. Mycol. Soc. **42,** 201–212.

Cavete, J.S. (1992). Function of Cutinolytic Enzymes in the Infection of Gerbera Flowers by *Botrytis cinerea.* Ph.D. Dissertation (Utrecht, The Netherlands: University of Utrecht), pp. 75–84.

Crawford, M.S., and Kolattukudy, P.E. (1987). Pectate lyase from *Fusarium solani* f. sp. *pisi*: Purification, characterization, *in vitro* translation of the mRNA, and involvement in pathogenicity. Arch. Biochem. Biophys. **258,** 196–205.

Dantzig, A.H., Zuckerman, S.H., and Andonov-Roland, M.M. (1986). Isolation of a *Fusarium solani* mutant reduced in cutinase activity and virulence. J. Bacteriol. **168,** 911–916.

Dickman, M.B., Patil, S.S., and Kolattukudy, P.E. (1982). Purification, characterization and role in infection of an extracellular cutinolytic enzyme from *Colletotrichum gloeosporioides* Penz. on *Carica papaya* L. Physiol. Plant Pathol. **20,** 333–347.

Dickman, M.B., Podila, G.K., and Kolattukudy, P.E. (1989). Insertion of cutinase gene into a wound pathogen enables it to infect intact host. Nature **342,** 446–448.

Hankin, L., and Kolattukudy, P.E. (1968). Metabolism of a plant wax paraffin (*n*-nonacosane) by a soil bacterium (*Micrococcus cerificans*). J. Gen. Microbiol. **51,** 457–463.

Kämper, J., Kämper, U., Rogers, L.M., and Kolattukudy, P.E. (1994). Identification of regulatory elements in the cutinase promoter from *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*). J. Biol. Chem. **269,** 9195–9204.

Kolattukudy, P.E. (1985). Enzymatic penetration of the plant cuticle by fungal pathogens. Annu. Rev. Phytopathol. **23,** 223–250.

Kolattukudy, P.E., Soliday, C.L., Woloshuk, C.P., and Crawford, M. (1985). Molecular biology of the early events in the fungal penetration into plants. In Molecular Genetics of Filamentous Fungi, W.E. Timberlake, ed (New York: Alan R. Liss, Inc.), pp. 421–438.

Kolattukudy, P.E., Podila, G.K., Roberts, E., and Dickman, M.D. (1989). Gene expression resulting from the early signals in plant-fungus interaction. In Molecular Biology of Plant-Pathogen Interactions, B. Staskawicz, P. Ahlquist, and O. Yoder, eds (New York: Alan R. Liss, Inc.), pp. 87–102.

Kolattukudy, P.E., Kämper, J., Kämper, U., González-Candelas, L., and Guo, W. (1994). Fungus-induced degradation and reinforcement of defensive barriers of plants. In Host Wall Alterations by Parasitic Fungi, O. Petrini and G.B. Ouellette, eds (St. Paul, MN: American Phytopathological Society), pp. 67–79.

Köller, W., Allan, C.R., and Kolattukudy, P.E. (1982a). Inhibition of cutinase and prevention of fungal penetration into plants by benomyl—A possible protective mode of action. Pesticide Biochem. Phys. **18,** 15–25.

Köller, W., Allan, C.R., and Kolattukudy, P.E. (1982b). Protection of *Pisum sativum* from *Fusarium solani* f. sp. *pisi* by inhibition of cutinase with organophosphorus pesticides. Phytopathology **72,** 1425–1430.

Köller, W., Allan, C.R., and Kolattukudy, P.E. (1982c). Role of cutinase and cell wall degrading enzymes in infection of *Pisum sativum* by *Fusarium solani* f. sp. *pisi.* Physiol. Plant Pathol. **20,** 47–60.

Kraft, J.M., Burke, D.W., and Haglund, W.A. (1981). *Fusarium* diseases of beans, peas, and lentils. In *Fusarium*: Diseases, Biology and Taxonomy, P.E. Nelson, T.A. Toussoun, and R.J. Cook, eds (University Park, PA: The Pennsylvania State University Press), pp. 142–156.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Maiti, I.B., and Kolattukudy, P.E. (1979). Prevention of fungal infection of plants by specific inhibition of cutinase. Science **205,** 507–508.

Miao, V.P.W., Matthews, D.E., and Van Etten, H.D. (1991). Identification and chromosomal locations of a family of cytochrome P-450 genes for pisatin detoxification in the fungus *Nectria haematococca.* Mol. Gen. Genet. **226,** 214–223.

Podila, G.K., Dickman, M.B., Rogers, L.M., and Kolattukudy, P.E. (1989). Regulation of expression of fungal genes by plant signals. In Molecular Biology of Filamentous Fungi, H. Nevalainen and M. Penttilä, eds (Helsinki, Finland: Foundation for Biotechnical and Industrial Fermentation Research), pp. 217–226.

Purdy, R.E., and Kolattukudy, P.E. (1973). Depolymerization of a hydroxy fatty acid biopolymer, cutin, by an extracellular enzyme from *Fusarium solani* f. *pisi*: Isolation and some properties of the enzyme. Arch. Biochem. Biophys. **159,** 61–69.

Purdy, R.E., and Kolattukudy, P.E. (1975a). Hydrolysis of plant cuticle by plant pathogens. Purification, amino acid composition, and molecular weight of two isozymes of cutinase and a nonspecific esterase from *Fusarium solani* f. *pisi.* Biochemistry **14,** 2824–2831.

Purdy, R.E., and Kolattukudy, P.E. (1975b). Hydrolysis of plant cuticle by plant pathogens. Properties of cutinase I, cutinase II, and a nonspecific esterase isolated from *Fusarium solani pisi.* Biochemistry **14,** 2832–2840.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Shaykh, M., Soliday, C.I., and Kolattukudy, P.E. (1977). Proof for the production of cutinase by *Fusarium solani* f. *pisi* during penetration into its host, *Pisum sativum.* Plant Physiol. **60,** 170–172.

Soliday, C.L., and Kolattukudy, P.E. (1976). Isolation and characterization of a cutinase from *Fusarium roseum culmorum* and its immunological comparison with cutinases from *F. solani pisi.* Arch. Biochem. Biophys. **176,** 334–343.

Soliday, C.L., Flurkey, W.H., Okita, T.W., and Kolattukudy, P.E. (1984). Cloning and structure determination of cDNA for cutinase, an enzyme involved in fungal penetration of plants. Proc. Natl. Acad. Sci. USA **81,** 3939–3943.

Stahl, D.J., and Schäfer, W. (1992). Cutinase is not required for fungal pathogenicity on pea. Plant Cell **4,** 621–629.

Sweigard, J.A., Chumley, F.G., and Valent, B. (1992). Disruption of *Magnaporthe grisea* cutinase gene. Mol. Gen. Genet. **232,** 183–190.

van den Ende, G., and Linskens, H.F. (1974). Cutinolytic enzymes in relation to pathogenesis. Annu. Rev. Phytopathol. **12,** 247–258.

Walton, T.J., and Kolattukudy, P.E. (1972). Determination of the structures of cutin monomers by a novel depolymerization procedure and combined gas chromatography and mass spectrometry. Biochemistry **11,** 1885–1897.